



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Wies Ter Laak, et al.

Serial No.: 10/608,095

Filed: June 30, 2003

Title: COMPOSITION COMPRISING COCOA

Art Unit: 1654

Examiner: Patricia Leith

March 15, 2005

DECLARATION UNDER RULE 132

Assistant Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Eline M. van der Beek, residing C. van Ommerenweg 10, 6711 JD Ede, the Netherlands,
do hereby declare that:

1. I am a citizen of the Netherlands,
2. My educational and technical background in the field of Neuroendocrinology is as follows:
 - a) I am a PhD from the Department of Cell Biology, Medical Faculty, Utrecht University, Utrecht, The Netherlands,
 - b) From 1st januari 1994 till 15th august 2000, I was employed at the Human and Animal Physiology Group of the Animal Sciences Department from the Wageningen University, The Netherlands,

- c) I have been employed by Nutricia N.V. since 15th august 2000, presently as research manager.
3. I have read Ter Laak et al., US application 10/608,095 filed June 30, 2003.
 4. I have investigated the effect of cocoa, *Cimicifuga racemosa*, and a combination of cocoa and *Cimicifuga racemosa* on basal prolactin release *in vivo*, as explained below.

Animals

Young adult male Wistar rats (n=26), weighing 350 gram, were housed in groups of 4 to 6 animals. After cannulation of the jugular vein, animals were housed individually. The animals were kept under a 12/12 LD cycle, lights on at 7 a.m. and given standard pelleted food and water *ad libitum*. To minimize possible effects of stress, bedding of all animals was mixed and a handful was added routinely to the fresh bedding with each cage change. The rats were handled and weighed regularly. In the week prior to blood sampling, rats were trained to eat custard.

Study design

The animals were allowed to habituate for 1 week after arrival, after which the animals received a jugular vein catheter. The rats were allowed to recover for at least 7 days before further experimentation. The animals were randomly assigned to the experimental groups in order to reach a group size of 8-11 animals for each component supplementation after 3 successive blood sampling procedures, once a week.

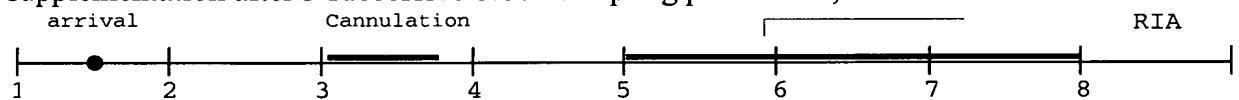


Figure 1: Study design

Cannulation of the jugular vein was performed according to the method of Steffens (Steffens, 1969), to allow stress-free blood sampling in freely-moving animals. Duplocilline® (0.01 ml; Mycofarm BV, De Bilt, the Netherlands) was administered s.c. 24 hrs prior to the surgery, and repeated at 48 hrs and 96 hrs after surgery. Cannulation was performed under isoflurane anaesthesia, and the cannula fixed to the skull using screws and dental cement using Xylocaine® 10% spray (Astra Pharmaceutica BV, Zoetermeer, the Netherlands) to numb the skull. The cannula was filled with 0.05 ml of a polyvinylpyrrolidone (PVP) solution, containing 500 U heparin per ml. Temgesic® (0.01 ml) was given for post-operative sedation, and repeated after 24 hrs. Two days after the operation, cannulas were flushed with heparinic saline and filled with fresh PVP solution.

Treatment

4 experimental mixtures of components in custard were prepared, which were randomly administered to the rats (n=11 for treatment). The experimental diets comprised:
Control diet (Ctrl): custard only

Cocoa Diet (CC): custard with 500 mg cocoa extract per kg body weight (*Theobroma cocoa*, Nattrop, San Leandro),

Cocoa + Cimicifuga (CC + CRlow): custard with a combination of cocoa extract (see above) and 10 mg *Cimicifuga racemosa* extract per kg of body weight. (*Cimicifuga racemosa* extract comprising 2.5 % triterpene glycosides. (Pureworld Botanicals, Inc., New Jersey)

Cocoa + Cimicifuga (CC + CRhigh): custard with a combination of cocoa extract (see above) and 100 mg *Cimicifuga racemosa* extract per kg of body (see above).

The mixtures were freshly prepared on each blood sampling day, by adding the components to 2 ml of strawberry custard (Campina, Zaltbommel, the Netherlands). The custards were served in small troughs made of glass and eaten by the rats voluntarily. This administration route was chosen to further minimize stress by administration of compounds since stress is known to induce short term PRL release (Mattheij *et al.*, 1978). An additional group of 3 animals was given a high dose of *C. racemosa* (200 mg/kg bwt) to verify the choice for the 2 dosages of the herb used.

Blood sampling

On each day of blood sampling, animals were connected to a blood sampling tube and PVP was aspirated and replaced by heparinized saline. The rats were allowed to habituate for 2 hrs before the start of the experiment. Before compound administration a blood sample was drawn (basal, t=0). subsequently, the selected compound was self-administered and after 15 min a second blood sample was taken (t=15). Subsequently, blood was sampled at t=30, t=60, t=120, and t=240 min (see figure 2). Six blood samples of 170 µl were drawn between 10 a.m. and 3 p.m.

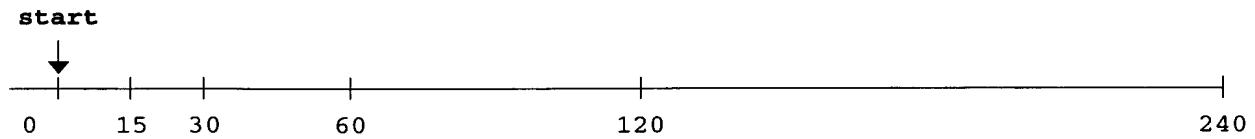


Figure 2: Blood sampling scheme.

Each blood sample was collected in heparinized (5 U/cup) Eppendorf tubes. The samples were centrifuged for 3 min at 13000 rpm. Plasma was kept at -20°C until further analysis.

Hormone determination

PRL levels were determined in triplicate by a double-antibody radioimmunoassay (RIA), using the rPRL-I-5 for labeling (obtained from the NIDDK), anti-rPRL-s-415 as antiserum (obtained from the Netherlands Cancer Institute, Dr. H.G. Kwa), and with Sac-cel® (donkey anti-rabbit, Welcome Reagents, Beckenham, UK) as a second antibody. The PRL concentration was expressed relative to the NIDDK-rPRL-RP-3 (AFP-4459B) reference. The intra-assay variations were determined using pooled rat serum and amounted to 12.7 and 15.7%, respectively.

Statistics

Data are presented as total PRL release during the sampling period (area under the curve, AUC \pm SEM). Statistical analyses was performed using SPSS for Windows (version 11.5). Normal distribution was checked by box plot analysis, and data were analyzed using ANOVA. The post hoc multiple comparisons tests Bonferroni and LSD were used when equal variances were found. Dunnett's T3 test was used when equal variances were absent. Results are considered significant when $p<0.05$.

References

Mattheij, J.A. and J.J. Swarts (1978). "Circadian Variations in the Plasma Concentration of Prolactin in the Adult Male Rat." *Journal of Endocrinology* 79: 85-89.

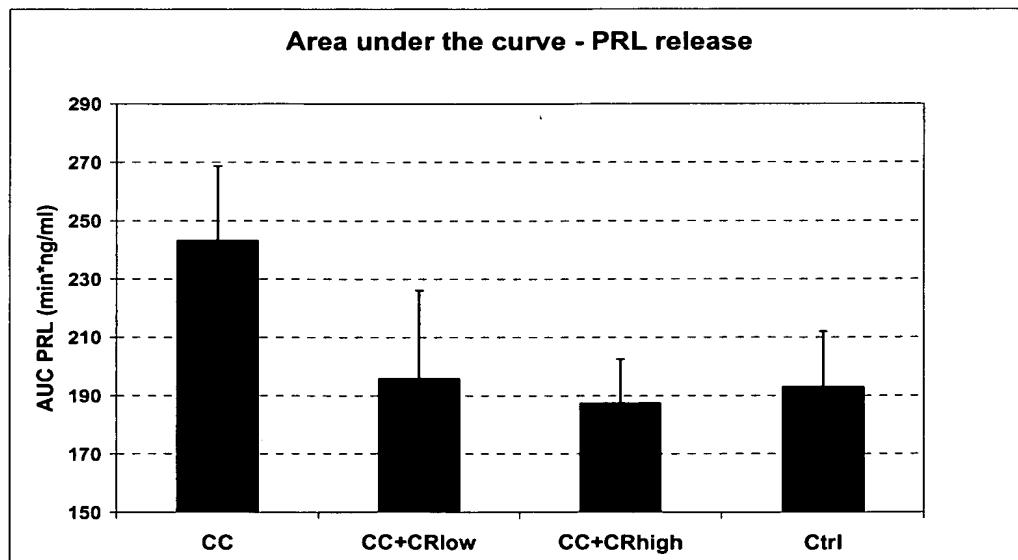
Steffens, A. B. (1969). "A Method for Frequent Sampling of Blood and Continuous Infusion of Fluids in the Rat Without Disturbing the Animal." *Physiology and Behavior* 4: 833-836.

Results

The control animals (custard only) showed no change in blood prolactin (PRL) release. The animals treated with cocoa showed an increase in PRL release compared to their baseline sample and the total amount of PRL released (i.e. area under the curve calculated as the cumulative value of PRL levels during the sampling period of 240 min) was increased compared to control animals. Coadministration of Cimicifuga racemosa extract (i.e. plant derived Dopamine d2 receptor agonist) and cacao reduced the total amount of PRL released during the sampling period compared to cacao alone.

Conclusion

These results are indicative for prolactin release tempering effect of Cimicifuga on cocoa induced prolactin release, and gives evidence of the beneficial and non-obvious effects of a combination of cocoa and Cimicifuga racemosa. Since Cimicifuga racemosa is a Dopamine D2 receptor agonist (Winterhof, 2000), it provides evidence of the advantageous effects of a combination of cocoa and a plant-derived dopamine D2 receptor agonist. *The results can be shown as follows (CC = Cocoa, CR = Cimicifuga racemosa): CC 242 ng/ml; CC + CRlow 196 ng/ml; CC + CRhigh 187 ng/ml; Control 194 ng/ml*



5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: Wageningen March 17th, 2005
(place) (date)

Signature:

E.M. van der Beek